SUPPLEMENTARY MATERIAL

Table S1. Relevant characteristics of the *E. coli* strains and plasmids used in this study

Strain or Plasmid	Relevant Characteristic(s)	Reference
E. coli strains		
TG1	Host strain used for routine cloning	
BL21 (DE3)	Strain used for overexpression of recombinant PBP5.	Life Technologies
Plasmids		
pGEM-T Easy	Plasmid used for initial cloning of PCR fragments; AMP^r.	Promega
pET28a(+)	Expression vector carrying T7lac promoter, adds an N-terminal His tag; KAN^r.	Novagen
pTEX4302.1	pET28a(+) derivative carrying a 1929 bp *pbp5-S* gene fragment from *E. faecium* Com15 lacking the first 108 bp of the PBP5 coding sequence, which correspond to the transmembrane domain region.	This study
pTEX2193.1	pET28a(+) derivative carrying a 1929 bp *pbp5-R* gene fragment from *E. faecium* C68 lacking the first 108 bp of the PBP5 coding sequence, which corresponds to the transmembrane domain encoding region.	This study

AMP, Ampicillin; KAN, Kanamycin; Superscript "r" designates resistance.

Table S2. Oligonucleotides used in this study

Primer Name	Sequence 5′- 3′	Relevant Characteristics
Recombinant PBP5 (rPBP5)		
F-rPBP5	GGAATTCCATATGCAA GAAACCCAAGCAGTA	Forward for expression of Δ1-36 rPBP5; NdeI site underlined.
R-rPBP5	CGGGATCCTTATTGATA ATTTTGGTTGAG	Reverse for expression of Δ1-36 rPBP5; BamHI site underlined.
Northern Blot Probe		
F-*pbp5*-218	GGCGAACTTCTAATTA ATCC	Forward for amplification of a 218 pb fragment of *pbp5* used a probe for northern hybridization.
R-*pbp5*-218	GGAATCCCTAAAGCAG AAAG	Reverse for amplification of a 218 pb fragment of *pbp5* used a probe for northern hybridization.
Primers to amplify upstream region of *pbp5* for all the strains included in this study		
*ftsW*3p-F	ATACAGGCCGAAGAGT TGCC	Forward for 3' end of *ftsW*.
pbp5-R-115-134[a]	CCAGCTTCTACTGCTTG GGT	Reverse for 5' end of *pbp5*
Primers to confirm the insertion of 3 genes upstream of *pbp5* in TX82 and 1.230.933 These primers were designed based on the upstream region of *pbp5* in the complete genome sequence of the *E. faecium* strain Aus0004.		
hisJ-F	ATCGCTAATTCCGTCACA CCT	Forward for *hisJ,* encoding a putative histidinol phosphate phosphatase (HisJ) (reverse based on the direction of the gene); when use with primer *pbp5*-R$^{115\text{-}134}$ gives a product of 978 bp.
gnat-F	TCAACGGCTCTATCTGC TCA	Forward for *gnat,* encoding a putative acetyltransferase; when use with primer *pbp5*-R$^{115\text{-}134}$ gives a product of 1294 bp.
pbp5-R-115-134[a]	CCAGCTTCTACTGCTTGG GT	Reverse for *pbp5,* located at position 115 to 134 from the *pbp5* start codon.
psr-F	TGCAGCTTACTCTTATG GGGG	Forward for *psr;* when use with primer IS*Efm*1-3p-R gives a product of 1460 bp.
IS*Efm*1-3p-R	AGCCCTTTAACAGAAC GTGAGT	Reverse for IS*Efm* (insertion sequence)*;* when use with primer *psr*-F gives a product of 1460 bp.

[a]Same primer

Table S3. Amino acid sequence in the 21 positions previously reported to vary between PBP5-S and PBP5-R of the subclade A2 *E. faecium* strains EnGen35, EnGen21 and EnGen52.

Strain	MIC (µg/ml)	PBP5-S/R type	24	27	34	66	68	85	100	144	172	177	204	216	324	466'	485	496	499	525	586	629	667
PBP5-S-Consensus	**≤2**		**V**	**S**	**R**	**G**	**A**	**E**	**E**	**K**	**T**	**L**	**D**	**A**	**T**	**-**	**M**	**N**	**A**	**E**	**V**	**E**	**P**
EnGen35	1	S_8/R_{13}	A	G	Q	E	A	E	Q	Q	A	I	D	S	A	-	M	K	T	D	V	E	P
EnGen21	8	S_8/R_{13}	A	G	Q	E	A	E	Q	Q	A	I	D	S	A	-	M	K	I[a]	D	V	E	P
EnGen52	128	S_8/R_{13}	A	G	Q	E	A	E	Q	Q	A	I	D	S	A	-	M	K	T	D	V	E	P
PBP5-R Consensus	**≥16**		**A**	**G**	**Q**	**E**	**T**	**D**	**Q**	**Q**	**A**	**I**	**G**	**S**	**A**	**S**	**A/T**	**K**	**T/I[a]**	**D**	**L**	**V**	**S**

[a] Isoleucine at position 499 is commonly found in strain from clade A2 with "hybrid-like" PBP5 sequences.

Table S4. Amino acid changes outside the 21 positions previously reported to vary between PBP5-S and PBP5-R of the subclade A2 *E. faecium* strains EnGen35, EnGen21 and EnGen52.

Strain	MIC (µg/ml)	39	314	401	406	509	606
PBP5-S-Consensus	**≤2**	**T**	**T**	**A**	**P**	**D**	**S**
EnGen35	1	N	T	A	P	D	S
EnGen21	8	T	T	S	P	D	S
EnGen52	128	N	I	A	A	E	F
PBP5-R Consensus	**≥16**	**T**	**T**	**A**	**P**	**D**	**S**

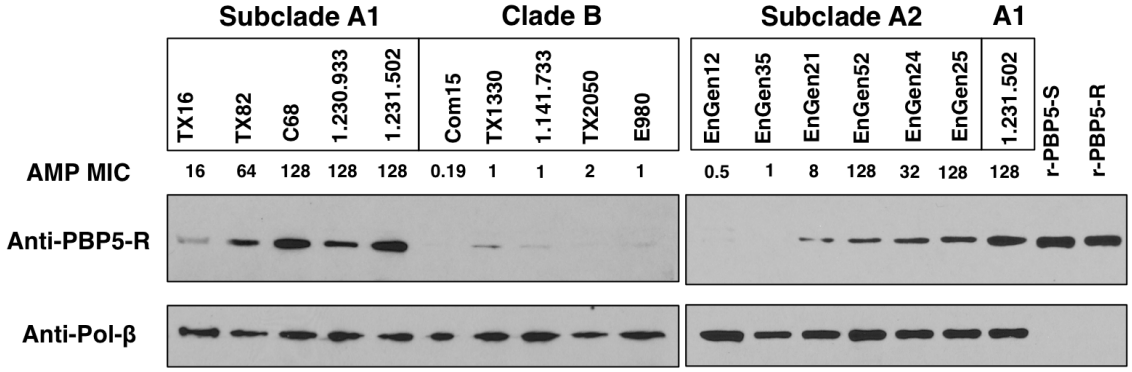

Figure S1. PBP5-R and RNA polymerase subunit β protein levels detected by Western blot. Equivalent protein samples were separated by SDS-PAGE, transferred to a PVDF membrane and detected using a polyclonal serum raised against r-PBP5-R from C68 or a monoclonal antibody against RNA polymerase subunit β protein. The *E. faecium* strains and ampicillin MICs are indicated above the image (See Table 1 for detailed description of the strains). Equivalent protein samples for strain $1.231.502_{A1}$ were loaded in the two gels for comparisons between gels; 10 ng of recombinant PBP5-S and PBP5-R were loaded into the last two lanes as controls.

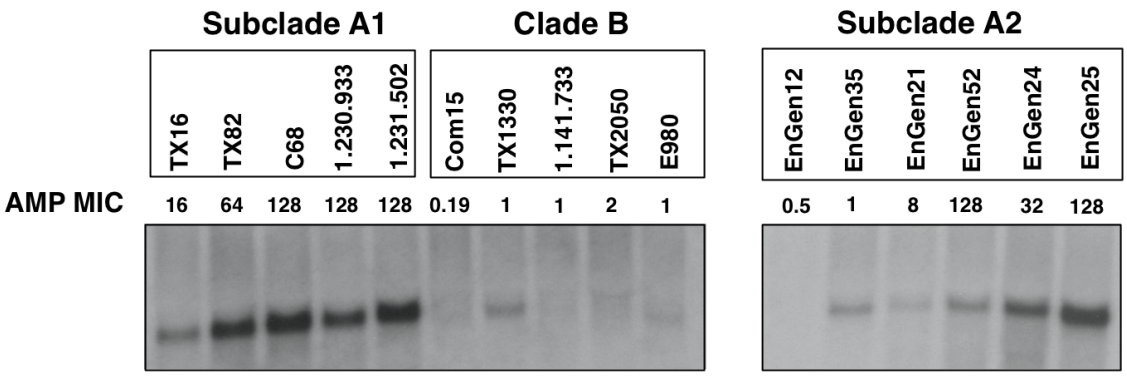

Figure S2. Differential *pbp5* mRNA levels in the *E. faecium* strains detected by northern hybridization. Total RNA was extracted from the 16 *E. faecium* strains grown in BHI to late exponential phase and hybridized with an internal probe of the *pbp5* gene. The *E. faecium* strains and ampicillin MICs are indicated above the image (See Table 1 for detailed description of the strains).

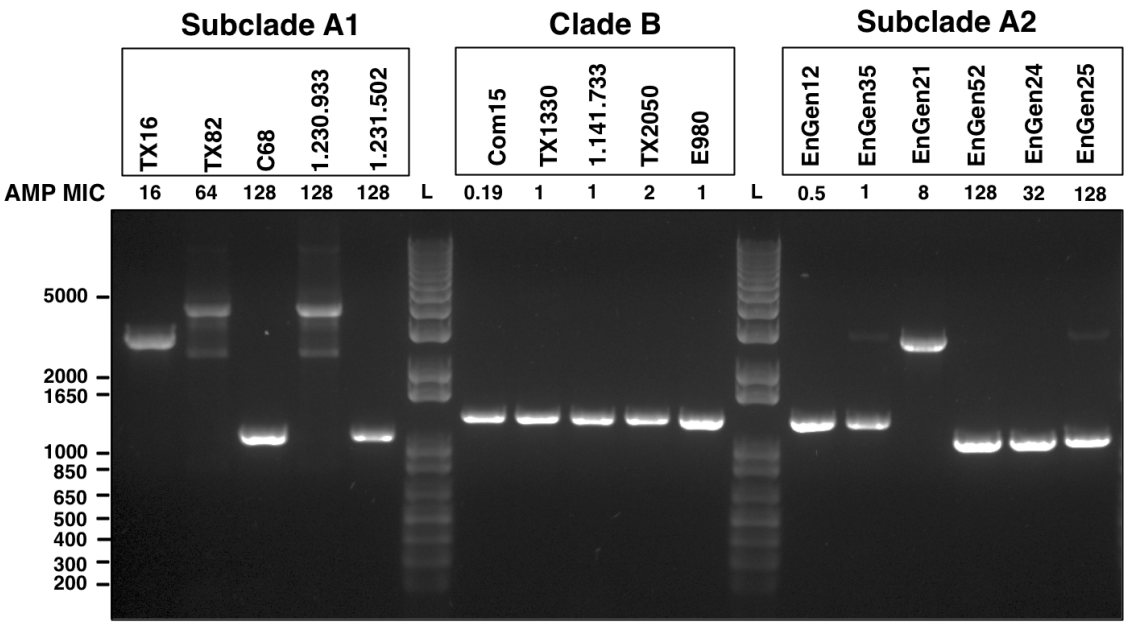

Figure S3. PCR upstream of *pbp5* using primers *ftsW*3p-F and *pbp5*-R-$^{115\text{-}134}$ that anneal on *ftsW* and *pbp5*, respectively. The *E. faecium* strains and ampicillin MICs are indicated above the image (See Table 1 for detailed description of the strains). L, DNA ladder.

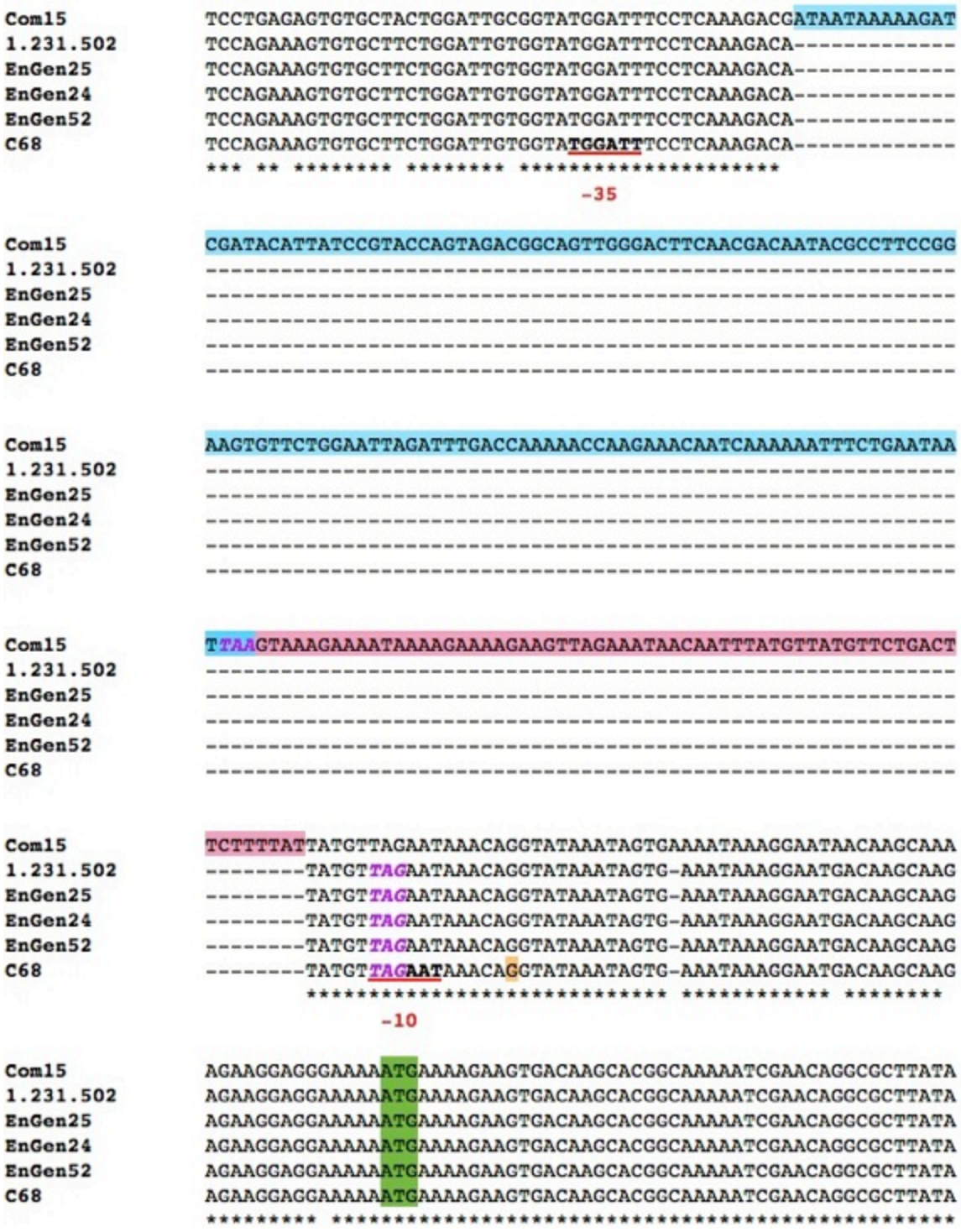

Fig. S4. Alignment of the upstream region of *pbp5* from the *E. faecium* strains 1.231.502$_{A1}$, EnGen25$_{A2}$, EnGen24$_{A2}$, EnGen52$_{A2}$ and C68$_{A1}$ against Com15$_{B}$.

The 201 bp region deleted is shown in light blue (corresponding to 137 bp of the 3' end of *psr*) and in pink (corresponding to the intergenic region between *psr* and *pbp5*). The transcriptional start site of the *pbp5* gene, demonstrated by Rice et al. and the predictive translational start codon are highlighted in yellow and green, respectively. The putative -10 and -35 boxes predicted in strain $C68_{A1}$ are in bold and underlined in red. The *psr* stop codon for the clade A strains that have deleted the 201 bp is shown in magenta and italics.